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Review

# Extracellular vesicles in gastrointestinal cancer in conjunction with microbiota: On the border of Kingdoms

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## ABSTRACT

Extracellular vesicle (EV) production is a universal feature of metazoan cells as well as prokaryotes (bMVs - bacterial microvesicles). They are small vesicles with phospholipid membrane carrying proteins, DNA and different classes of RNAs and are heavily involved in intercellular communication acting as vectors of information to target cells. For the last decade, the interest in EV research has exponentially increased though thorough studies of their roles in various pathologies that was not previously possible due to technical limitations. This review focuses on research evaluating the role of EV production in gastrointestinal (GI) cancer development in conjunction with GI microbiota and inflammatory diseases. We also discuss recent studies on the promising role of EVs and their content as biomarkers for early diagnosis of GI cancers.

The bMVs have also been implicated in the pathogenesis of GI chronic inflammatory diseases, however, possible role of bMVs in tumorigenesis remains underestimated. We propose that EVs from eukaryotic cells as well as from different microbial, fungi, parasitic species and edible plants in GI tract act as mediators of intracellular and inter-species communication, particularly facilitating tumor cell survival and multi-drug resistance.

In conclusion, we suggest that matching sequences from EV proteomes (available from public databases) with known protein sequences of microbiome gut bacteria will be useful in identification of antigen mimicry between evolutionary conservative protein sequences. Using this approach we identified *Bacteroides* spp. pseudokinase with activation loop and homology to PDGFRα, providing a proof-of-concept strategy. We speculate that existence of microbial pseudokinase that 'mimics' PDGFRα may be related to PDGFRα and *Bacteroides* spp. roles in colorectal carcinogenesis that require further investigation.

*Abbreviations:* AchE, acetylcholinesterase esterase; Ago, Argonaute protein family, essential catalytic components of RISC; AnV, annexin V; APC, antigen-presenting cells; ARF6, ADP-ribosylation factor 6; bft, *Bacteroides fragilis* toxin; bMVs, bacterial microvesicles; BPD, benign pancreatic disease; CEA, carcinoembryonic antigen; CEC, colonic epithelial cells; CrD, Crohn's disease; CRC, colorectal cancer; DAPI, (4′,6-diamidino-2-phenylindole); DHR, dihydrorhodamine; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; EpCAM, epithelial cell adhesion molecule; EVs, extracellular vesicles; FFPE, formalin-fixed paraffin embedded; FP, fluorescent protein; GI, gastrointestinal; GM1, monosialotetrahexosylganglioside; GPC1, membrane-anchored proteoglycan molecule glypican-1; IBD, inflammatory bowel disease; IEM, immunoelectron microscopy; IFC, imaging flow cytometry; IL, interleukin; KRAS, GTPase, that in human is encodes by the *KRAS* gene; lnsRNAs, long non-coding RNAs; LSPR, localized surface plasmon resonance; MEK-ERK, mitogen-activated protein kinase kinase/extrac membrane-anchored proteoglycan molecule glypican-1ellular-signal regulated kinase; MPs, microparticles; MUC1, mucin1; MVs, microvesicles; MVBs, multivesicular bodies; OD, optical density; OMPs, outer membrane vesicles; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PCA, capsular polysaccharide A; PCD, programmed cell death; PDAC, pancreatic ductal adenocarcinoma; PDGFR, platelet-derived growth factor receptor alpha; PE, phycoerythrin; PI, propidium iodide; PODO, podoplanin; qRT-PCR, quantitative RT-PCR; RFP, red fluorescent protein; RISC, RNA-induced silencing complex; SCID, severe combined immunodeficiency (non-human); SELN, exosome-like synthesized nanoparticles; SMLM, single-molecule localization microscopy; TEV, tumor-originating extracellular vesicles; TNF-alpha, tumor necrotic factor alpha; TF, tissue factor; YSPAN8, tetraspanin 8; VEGF, vascular endothelial growth factor; VTEs, venous thromboembolic events

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## 1. Introduction

Chronic inflammation pathologies of GI such as inflammatory bowel disease (IBD), Crohn's disease (CrD), *Helicobacter pylori*-associated inflammation and chronic pancreatitis have been identified as strong risk factors for cancer development [1–6]. The initial hypothesis that the emergence of the tumor is associated with chronic inflammation was postulated by Rudolf Virchow in 1863 [7]. Today, the causative link between cancer and chronic inflammation is widely accepted, though molecular and cellular mechanisms of this association have not been resolved [8]. About 15% of the global cancer burden can be attributed to infectious agents [9], of which chronic inflammation is a major component [1]. Acute inflammation is self-limiting, since the production of anti-inflammatory cytokines (IL-1, IL-10, IL-13 etc.) follows the production of pro-inflammatory cytokines (IL-1, TNF-alpha, IFNγ etc.) [8]. The strongest association of chronic inflammation and underlying infection with cancerogenesis is found between inflammatory bowel diseases and colon cancer, *Helicobacter pylori* and gastric cancer, hepatitis C and liver carcinoma, schistosomiasis and bladder and colon carcinomas [10]. The broader implication of these observations was that chemokines and other cytokines are widely involved in cancer development, however, the detailed mechanisms linked to infection and inflammation in relation to cancer are not well understood.

It is well known that cell activation and pathogenesis of a variety of diseases are often associated with increased levels of EVs released in body fluids including plasma, liquor, urine, bile, saliva, semen, vitreous and synovial fluids, atherosclerotic plagues, mucus and intestinal fluids, ascitic and pleural fluids [11–14]. EVs production by eukaryotic cells is upregulated during cell activation and growth, thus playing an essential role in cellular communication during cancer development. However, until recently it was not clear how commensal and pathogenic bacteria, and members of gut microbiota, communicate with other microbial and eukaryotic cells and the immune system. Studies during the last decade that are focusing on the association between gastrointestinal cancer and inflammatory diseases with certain types of bacterial infections often are overlooking the intensive production of outer membrane vesicles (OMPs) by different types of gastrointestinal bacterial commensals and pathogens as well as fungi, parasites invading the GI tract, and nematodes.

This review focuses on research assessing EVs originating from different types of eukaryotic and prokaryotic cells inside the GI tract which separates symbionts and commensals in our bodies. We also discuss the role of EVs originated from GI cancers as potential biomarker tools. Early diagnosis of GI cancers continues to be a major challenge with a miss rate of up to 6.7% - for the upper GI tract with endoscopy and colonoscopy [15] and up to 6% miss rate for colorectal cancers [16]. Due to the invasiveness of these procedures and the possibility of complications [17], there is a high need for robust circulating biomarkers of GI cancers and effective non-invasive monitoring. In conclusion, we suggest that matching sequences from EV proteomes (available from public databases) with known protein sequences of microbiome gut bacteria will be useful in identification new molecular mimicry target protein sequences and provide a proof-of-concept strategy identifying *B. fragilis* pseudokinase that 'mimic' PDGFRα.

## 2. Extracellular vesicles (EVs) structure and biogenesis

Generally, three different classes of EVs (Fig. 1) are produced by metazoan cells, namely, exosomes, microparticles (MPs) or microvesicles, sometimes also named ectosomes, and apoptotic bodies. These EVs are distinguished by size, their content, morphology and by different mechanisms of their biogenesis [18–19]. Moreover, it is well accepted that production of EVs represents a universal feature of life, since gram-negative and gram-positive bacteria as well as *Archaea* generate outer membrane vesicles [20–24]. It is believed that the formation of microparticles (MPs) usually happens via plasma membrane budding and shedding associated with membrane sites enriched with lipid rafts [25–26,18]. Unlike MPs, exosome formation occurs via rerouting of multivesicular bodies (MVBs) to the cell surface [27–28], where they fuse with the cell membrane and exit the cell through exocytosis (detailed rev. [29]). As a result, exosomes are enriched with endosome- and MVBs-associated proteins, such as tetraspanins (CD9, CD63, CD81 and CD82 and CD151; whereas CD37, CD53 and Tssc6 are restricted by hematopoietic cells) [30–31]. CD63 is important for exosomal secretion, as the reduction of exosomal production was observed in CD63-knockout HEK-293 cells. However, at the same time no reduction in the secretion of microparticles > 150 nm size was noted, supporting the observation that CD63 is important for exosomal

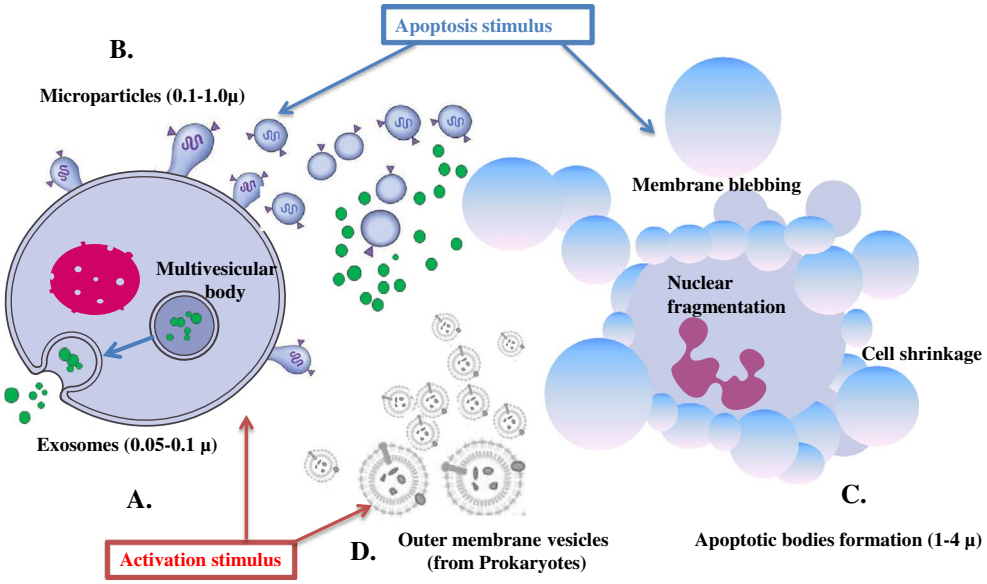


**Fig. 1.** Production of different classes of EVs. (A) Microparticles; (B) exosomes; (C) apoptotic bodies; (D) outer membrane vesicles.

**Table 1**
Circulating exosomes and microvesicles (below 200 nm) in patients with GI cancers.

| Cancer type | Cancer subtype | EVs source | EVs size | Detection and purification methods | Subpopulations of EVs | Potential biomarkers | Ref. |
|---|---|---|---|---|---|---|---|
| Colorectal cancers | Colorectal carcinoma—adenoma—NC | Human serum | ND | ExoQuick™ | Exosomal fraction from ExoQuick™ | miR-21, miR-291a, miR-92a | [66] |
| | CRC | Human serum | App. 50 nm (TEM) | Ultracentrifugation and Exosome Isolation Kit (Invitrogen), TEM, microRNA microarray, qRT-PCR | Exosomal fraction | miR-17-92a (recurrence biomarker) | [67] |
| | CRC | Human serum and cell lines FHC, HCT116, HT-29, RKO, SW48, SW480 | | Ultracentrifugation, Western blotting (CD81), miRNA microarray, qRT-PCR | EX fraction | 7 miRNA (let7a, miR-1220, miR-1246, miR-150, miR-21, miR-223, miR-23a) | [68] |
| | CRC | Serum samples | App. 100 nm | ExoScreen, NTA, ELISA | CRC-derived EVs | CD147, CD9 | [69] |
| | CRC | Human plasma and HCT116 cell line | < 220 nm | 0.22 μm filtration, Ultracentrifugation and $CD63^+$ beads isolation, FCM,RT-PCR, western blotting | $CD63^+$, EpCAM fractions of CRC-derived EVs | TSAP6 and p21 mRNA | [70] |
| | CRC | Human ascites | 30–150 nm | Western blot, nano-LC-MS | Ascite-derived EVs | CEACAM5, CD97, tetraspanins, plexin, TACSTD1, trophoblast glycoprotein | [50] |
| | CRC | Human plasma | 50–100 nm | FCM, Western blot, IEM | $FasL^+$, $TRAIL^+$, $CD63^+$ and $CEA^+$ CRC EVs | CEA and CD63 | [71] |
| Gastric cancer | Gastric cancer, TNM stages I-IV | Human serum | ND | ExoQuick™ | Exosomal fraction (ExoQuick™) | miR-10b-5p; miR-132-3p; miR-185-5p; miR-195-5p; miR-20a-3p; miR-296-5p | [72] |
| | Gastric adenocarcinoma | Peritoneum lavage fluid | ND | Microarray, qPCR | Exosomal fraction | miR-21; miR-1225-5p | [73] |
| | Gastric cancer (pre- and post-operative) | Paired plasma samples | 100 nm (TEM) | TEM, qRT-PCR | Exosomal fraction from "Total Exosome Isolation kit" (Invitrogen) | Long intergenic non-protein-coding RNA 152 (LINC00152) | [74] |
| | Gastric cancer | Human plasma | ND | FISH, qRT-PCR, ExoQuick™ | Exosomal fraction (ExoQuick™) | Five-miRNA signature; miR-185 associated with TNM stage | [75] |
| | | Ascites | | | Exosomal fraction | | [76] |
| Gastrointestinal stromal cancer | Gastrointestinal stromal tumors (GIST) | GIST solid tissues | 30–200 nm | STEM | Exosomes, microvesicles, spherosomes (multivesicular spheres) | ND | [77] |
| Esophageal cancers | Esophageal squamous cell carcinoma | Human serum | ND | ExoQuick™; Exiqon miRNA panel; qRT-PCR | Exosomal fraction (ExoQuick™) | Five-miRNA signature (miR-20b-5p; miR-28-3p; miR-192-5p; miR-223-3p; miR-296-5p) | [78] |
| | Esophageal squamous cell carcinoma | Human serum | ND | Differential centrifugation; TaqMan OpenArray miRNA Profiling; immunoblotting; | Exosomal fraction | miRNA-21 | [79] |
| | Esophageal squamous cell carcinoma; control group (esophageal achalasia and reflux esophagitis) | Human plasma | | Exosome isolation kit (Invitrogen); AChe activity; NTA | Exosomal fraction | Exosomal (or EVs) number in peripheral blood as prognostic biomarker | [80] |
| | Advanced esophageal carcinoma (HC and Barrett's esophagus) | Human serum | ND | ExoQuick™; miRNA profiling | Exosomal fraction | Multi-miRNA panel (RNU6-1/miR-16-5p, miR-25-3p/miR-320a, let-7e-5p/miR-15b-5p, miR-30a-5p/miR-324-5p, miR-17-5p/miR-194-5p | [81] |
| | Advanced Esophageal adenocarcinoma vs T2-T3 adenocarcinoma and squamous carcinoma | Human serum | ND | ExoQuick™; qRT-PCR; microRNA array, ELIZA | Exosomal fraction (ExoQuick™) | miR-223-5p, miR-483-5p | [82] |
| | Esophageal adenocarcinoma | Human serum | < 100 nm | ExoQuick™; qRT-PCR;TEM, Western blotting | Exosomal fraction (ExoQuick™) | miR-21 | [83] |
| | Barret's esophagus metaplasia-dysplasia-adenocarcinoma | Esophageal solid tissue samples | 90–200 nm | TEM | Matrix EVs | N/A | [84] |
| Intestinal cancer | Large bowel cancer | Human serum | App. 100 nm | IEM, TEM, AChEasy assay, qRT-PCR; Western blotting, ELISA | Exosomal fraction from ultracentrifugation | HSP60 | [85] |
| Pancreatic cancers | Pancreatic ductal adenocarcinoma (PDAC) | Human plasma before/after surgery | 60–120 nm | EM, LS-MS/MS, RT-PCR, ELISA | Exosomal fraction from ultracentrifugation | miRNA signature (high miR-10b, miR-21, miR-30c, miR-181, low miR-let7a); glypican-1 (GPC1); CA-19-9 | [86] |
| | Pancreaticobiliary cancer and PDAC | Pleural effusion and | Peak at 128 nm | TEM and SEM, NTA, FCM, western | Exosomal fraction from | N/A | [87] |

*(continued on next page)*

**Table 1** *(continued)*

| Cancer type | Cancer subtype | EVs source | EVs size | Detection and purification methods | Subpopulations of EVs | Potential biomarkers | Ref. |
|---|---|---|---|---|---|---|---|
| | | plasma-derived | | blotting, NGS | ultracentrifugation | | |
| | PDAC | Solid tissue and plasma | App. 100 nm | EM, FCM, NTA | PDAC-derived EVs | $MIF^+$ | [88] |
| | Pancreatic cancer | Human serum | App.100 nm | NTA, EM, CM, SEM | | CA-19–9, TSG101 | [89] |
| | PDAC | Human plasma | N/A | LSPR, TEM | PDAC-derived EVs | miR-10b | [90] |
| | Pancreatic cancer, PDAC | Solid tissue and plasma | < 100 nm | EM, FCM, ULPS-MS, RT-PCR | PDAC-derived EVs | $GPC1^+$ | [91] |
| | Pancreatic cancer | Human blood samples | 30–100 nm | EM, ExoChip | Pancreatic cancer (PC)-derived EVs | CD63 and Rab5 | [92] |
| | Pancreatic cancer | Serum samples | N/A | RT-PCR, qRT-PCR | PC-derived EVs | S-Exo PaCIC markers | [93] |
| | PDAC | Human serum | < 220 nm | RT-PCR | PC-derived EVs | miR-21; miR-17-5p | [94] |

secretion only [32]. Heat shock proteins HSP 70/90 are also enriched in exosomes, but not in shedded MPs [33]. The exosomal membrane is rigid with rigidity increasing from pH 5 to 7 as demonstrated by membrane fluidity probes (diphenylhexatriene), which is a result of increased amounts of desaturated phosphatidylcholines (PC), phosphatidylethanolamine, sphingomyelin, cholesterol [34] and ceramides [35]. Circulating exosomes are resistant to lipolytic enzymes and can stay for up to two weeks in the lymph nodes [36]. MPs are much more heterogeneous in size and structure, spanning from 0.1 to 1.0 μm, whereas exosomes are typically homogenous in size (0.04–0.1 μm) and shape [37,12]. Cells secrete exosomes and MPs simultaneously, but their ratios differ depending on the cell type and cell status [38–39].

Though their biogenesis is different and sizes are overlapping, separation and purification of these two major classes of EVs has been hampered by the limitations of existing methods [40–42]. Currently, various strategies including differential ultracentrifugation, filtration, density gradient separation, immunoaffinity, bead purification, and size exclusion chromatography have been used for isolation of different EV fractions, however, no universal approach exists. Limitation factors for ultracentrifugation include co-sedimentation of protein aggregates and co-purifying of non-specifically bound proteins [40]. The most straightforward approach for separating exosomes and MPs is FACS-based sorting accomplished by sorting similarly sized viral particles [43]. However, FACS-based cell sorting of EVs normally leads to size-based selection, could also be biased by the choice of antigens selected for FACS-sorting.

Both major EV classes contain different protein, RNA and DNA cargo [44–47], and also significantly differ by their lipid content. EVs may transfer information from the host cell to various target cells by direct cell-to-cell contact or alternatively, through secretion of soluble mediators and effectors [48–49]. Moreover, EVs may interact with target cells via surface-bound ligands, transferred surface receptors and membrane-associated enzymes, or deliver cytoplasmic or membrane-associated constituents, such as cytosolic proteins, different classes of RNA molecules, DNA, bioactive lipids and even cellular organelles such as mitochondria [11,14]. Several studies indicate that tumor-derived extracellular vesicles (TEVs) harbor signaling proteins that affect cell metabolism, mRNA processing, angiogenesis, and cell growth and motility, in addition to molecules that are likely required for TEV biogenesis [50,28]. It has been demonstrated that at least in some cases cellular proteins are selectively integrated into TEVs via ARF6-regulated endosome recycling, where the expression and activation of ARF6 is associated with an increase of tumor-invasive potential [51,52]. Plasma membrane-derived EVs, secreted from the parent cell, strengthens some, but not all plasma membrane proteins and those that integrated inside EVs conserve the topology of the parent cell plasma membrane [12].

The function of EVs appears to be dependent on their cargo. EVs shed from various tumor-cell lines have been thought to facilitate extracellular matrix (ECM) invasion, evasion of the immune response [53–54,37] and potentiate formation of metastasis in PDAC and other cancers [55–57]. Shedding of tumor-derived EVs can stimulate metastasis spreading, tumor-stroma interactions and angiogenesis [55,58–62]. EVs contain complex sets of cargo, depending on the physiological conditions in which they are generated and released, and once shed may impact a variety of cellular processes and modulate inflammatory response. The major question that remains to be elucidated is how specific cargoes are selectively absorbed and accumulated by different classes of EVs. It has been shown that posttranslational modifications of proteins with acyl, myristoyl, palmitoyl or glycophosphatidylinositol anchors may facilitate recruitment of specific proteins to EVs [11,63–64]. However, since recruiting of proteins happens also in the absence of these post-translational modifications, there are other yet unknown mechanisms that participate in protein cargo selection by EVs. The mechanisms of RNA and miRNA selection by EVs are poorly

**Table 2**
Circulating microvesicles (between 200 and 2000 nm) in gastrointestinal cancers.

| Cancer type | Cancer subtype | EVs source | EVs size | Detection and purification methods | Subpopulations of EVs | Potential biomarkers | Ref. |
|---|---|---|---|---|---|---|---|
| Colorectal cancers | CRC and PC | Human serum | ND* | FCM | $EpCAM^+$, $EpCAM^+CD147^+$, $CD147^+$ EpCAM-MVs | Increased $EpCAM^+$ $CD147^+$ | [95] |
| | CRC | Platelet-poor plasma | ND | FCM | CRC-derived MVs | ND | [96] |
| | CRC | Solid tissue (surgically or endoscopically resected) | < 1000 nm | RT-PCR, NTA | CRC-derived MVs | miR-1246 | [97] |
| | CRC | Human plasma | < 1000 nm | FCM | TEVs | ND | [98] |
| | Colon cancer | Bone marrow | ND | FCM | $CD34^+$/$HER2/neu^+$ stem cell EVs | ND | [99] |
| | Colon cancer | Human body fluids (pleural liquid) | 20–2000 nm | SEM | MVs, PEVs | ND | [100] |
| | CRC | Human plasma | < 1000 nm | FCM | $TF^+$, $PS^+$ EVs | D-dimer to detect VYE in CRC patients | [101] |
| Gastrointestinal cancers | Gastric cancer, III-IV stage | Human plasma | ND | Centrifugation, FCM, procoagulant activity | Leukocyte-derived MVs, Er-derived MVs, Endo-derived MVs, $PS^+$ MVs | Leukocyte MVs, Er MVs, Endo MVs > > In GC III-IV patients, $PS^+$ MVs | [102] |
| | Gastric cancer | Human plasma | 10–800 nm | TEM, FCM, AFM | TEVs, PEVs | CCR6 and HER2/neu | [103] |
| | | Human plasma | ND | FCM | Circulating tumor-derived EVs | ND | [104] |
| | Gastric cancer | Human platelet | ND | FCM | PEVs | ND | [105] |
| Pancreatic cancers | Human pancreatic adenocarcinoma cell lines BxPc-3 and Low.6pl | $TF^+$ MVs from PC injected in Par4-deficient mice | ND | FCM | $TF^+$ MVs | $TF^+$ MVs for PC-associated thrombosis | [106] |
| | CRC, PC, IBD | Platelet-poor plasma | ND | FCM | PMPs, EMPs, Er-derived MVs, LeuMVs, $TF^+$, $Fibrin^+$, $CEA^+$, $CA19–9^+$ MVs | Specific signature | [96] |
| | Pancreatic cancer with associated thrombosis | | ND | FCM | CD31, CD68, $AnnV^+$ $TF^+$, total $AnnV^+$, $AnnV^+$ $Muc1^+$ | $TF^+$ MVs for PC-associated thrombosis | [107] |
| | Pancreatic cancer with associated thrombosis (IVC-stenosis mice model) | Panc02 pancreatic ductal cell line induced in C57Bl/6 mice | ND | Centrifugation, FCM | $TF^+$ | ND | [108] |
| | Pancreatic carcinoma | Human blood | 200–2000 nm | SEM | MVs, PEVs | ND | [100] |

* ND – > 300 nm in the studies where conventional flow cytometry (FCM) was used for characterization of MVs (standard limit of sensitivity for conventional flow cytometer).

**Table 3**
Characterization of EVs derived from human cell lines.

| Cancer type | Cancer subtype | EVs source | EVs size | Detection and purification methods | Subpopulations of EVs | Potential biomarkers | Ref. |
|---|---|---|---|---|---|---|---|
| Colorectal cancers | Colorectal carcinoma | HCT-8 | ND | TEM | Fraction obtained with exosome-precipitation solution | miR-210 | [109] |
| | Colorectal carcinoma | SW480 | ND | 0.22 mkm filtration and differential ultracentrifugation; western blotting, microarray, functional assays, qRT-PCR | Fraction obtained from 48 h cultural supernatant | Erk1/2 kinase dependent internalization | [110] |
| | Colorectal carcinoma | SW480 | ND | Differential ultracentrifugation; single molecule-localization microscopy (SMLM), real-time PCR, FCM, western blotting | 48–72 h exosomal (EX) fractions | miR31 | [111] |
| | Colorectal carcinoma and pancreatic carcinoma | SW480, A818.4, Capan1 | ND | Differential ultracentrifugation; FCM, zymography, RT-PCR | | CD44v6 | [112] |
| | Colorectal carcinoma | HT-29, Caco-2 (in vivo mouse model) | 30–150 nm (TEM) | Differential ultracentrifugation and EX purification kit; TEM, FCM, western blotting (HSP70), qRT-PCR, functional assays | EX fraction from 24 and 48 h supernatant | CXCR4-axis | [61] |
| | Colorectal carcinoma | LIM1863 | Different 30–100 nm, 30–1000 nm fractions | Sequential ultrafiltration (0.1, 0.22, 0.45, 0.65 mkm), cryo-EM | EX and MVs fractions | Different proteomic signatures for EX and MVs | [113] |
| | Murine colorectal carcinoma | CT26 murine colorectal carcinoma associated with mouse macrophage Ana-1 cell line | | Differential ultracentrifugation, ExoQuick™; TEM, NTA, western blotting, mass-spectrometry, qRT-PCR | EX fraction obtained from cultural supernatant of MF associated with gastric cancer cells | Proteomic signatures | [114] |
| | Colorectal carcinoma | DLD-1 and DLD-1/5FU | 50–450 nm | NTA, RT-PCR | DLD-1 and DLD-1/5FU-derived MVs | miR-34a, miR-145 | [115] |
| | Colorectal carcinoma | LIM1863 | 50–150 nm (EX) and 100–1500 nm (MVs) | Ultracentrifugation and column separation; qRT-PCR, western blotting (CD9), deep RNA sequencing | A33-EX and EpCam EX and MVs | 32-miRNA signature | [116] |
| | Colorectal carcinoma | HCT-116 | ~100 nm | Differential ultracentrifugation; TEM, NTA, Ache assay, western blotting | $CD9^+$ and $CD63^+$ exosomal fraction | | [117] |
| | Colorectal carcinoma | DLD-1, WiDr, SW480, and COLO201 | 30–200 nm | NTA, western blotting | DLD-1, WiDr, SW480, and COLO201 exosomes | miR-1246, TGF-β | [97] |
| | Colorectal carcinoma | HCT116, HCT15, HT29, COLO201, COLO205, WiDr, SW1116 | ~100 nm | ExoScreen, immunoblot, NTA | HCT116, HCT15, HT29, COLO201, COLO205, WiDr, SW1116 exosomes | CD147/CD9, CA19-9 | [69] |
| | Colorectal carcinoma | LIM1215 | < 100 nm | Ultracentrifugation and 0.1 mkm filtration; immunoprecipitation, western blotting, LC-MS/MS | LIM1215-derived fraction | Cadherin17 | [118] |
| | Colorectal carcinoma | Sw480 and SW620 | 40–130 nm | EM, Cryo-EM, GeLC-MS/MS | Sw480, SW620 secretome and exosomes | TSG101, Alix, CD63, MET, S100A8, S100A9, TNC, FNB2, EGFR, JAG1, SRC, TNIK, CAV1, FLOT1, FLOT2, PROM1 | [119] |
| | Colorectal carcinoma | HT29 | 90 nm to 812 nm | NTA, TEM, FCM, western blotting | HT29 cell line-derived EVs | CEACAMs | [120] |
| | Colorectal carcinoma | Murine LIM1863 | ~100 nm (TEM) | Ultracentrifugation, combined with filtration; hA33/EpCam immunoaffinity purification, western blotting (TSG101, Alix, EpCam, A33), TEM, Gel C-MS/MS) | EX immunoaffinity purified fraction | | [121] |
| | Colorectal carcinoma | HCT15, SW480 and WiDr CRC | < 220 nm | SDS-PAGE, western blotting, RT-PCR | HT15, SW480 and WiDr-derived EVs | CD63, CD9, and CD81, miR-21 | [122] |
| | Colorectal carcinoma | SW480 and SW620 | 23 to 636 nm and 26–574 nm defined by NTA) | TEM, Nano-LC-ESI-MS/MS, NTA, peptide OFFGEL fractionation | SW480 and SW620-derived EVs | Tetraspanin and their associated proteins, FSCN1, STRAP, S100A4, S100A14 expression | [123] |
| | Colorectal carcinoma | HCT116 | ND | FCM, RT-PCR, western blotting | HCT116 EX fraction | TSAP6 | [70] |
| | Colorectal carcinoma | SW948, SW620, SW480, HT29, CaCo2 | ND | MALDI-TOF/TOF-MS, nano-HPLC/ESI-MS/MS, 2DGE | SW948, SW620, SW480, HT29, CaCo2 secretomes and EX fraction | Glod4, Rad23bC-terminal fragment of agrin, | [124] |
| | Colorectal carcinoma | LIM1215 | < 200 nm | Filtration and differential centrifugation, and immunocapture purification (A33); TEM, IEM (CD63), LS-MS/MS | A33 EX-fraction | Proteome analysis | [125] |
| | Colorectal carcinoma and | CX2 and Colo357 | < 200 nm | Filtration and Differential centrifugation; IEM | EX fraction | $HSP70^+$ $Bag4^+$ active fraction | [126] |

*(continued on next page)*

**Table 3** *(continued)*

| Cancer type | Cancer subtype | EVs source | EVs size | Detection and purification methods | Subpopulations of EVs | Potential biomarkers | Ref. |
|---|---|---|---|---|---|---|---|
| | pancreas carcinoma | | | (HSP70), western blotting, FCM, functional assays | | | |
| | Colorectal carcinoma | SW480 | 40–150 nm | Microarray, TEM, IHC, RT-PCR | SW480 CRC cell line-derived EX fraction | CENPE, KIF15, CEP55, CCNA2, NEK2, PBK, and CDK8 | [119] |
| | Colorectal carcinoma | SW403 and CRC28462 | 50–100 nm | FCM, western blotting, IEM | $FasL^+$, $TRAIL^+$, $CD63^+$ and $CEA^+$ CRC MVs | CEA and CD63 | [71] |
| | Colorectal carcinoma | HT29-19A and T84-DRB1*0401/CIITA | 30–90 nm | IEM, FCM, MALDI-TOF-MS | Apical and basolateral EX fraction | CD63 and CD26 | [127] |
| Esophageal cancers | Esophageal squamous cell carcinoma | EC9706 | ND | Differential centrifugation | EX fraction from 48-h cell supernatant | | [79] |
| | Esophageal squamous cell carcinoma | TE2; Murine SCCVII | ND | Exosome-isolation kit (Invitrogen); qRT-PCR; AchE quantitation | CD63-GFP-tagged exosomes from TE2 | CD63 | [80] |
| | Esophageal carcinoma | EC9706 | 30–60 nm | TEM, Solexa, RT-PCR | EC9706 EX fraction | let-7 family, miR-21-5p, miR-26a-5p | [128] |
| | Co-culture esophageal carcinoma and fibroblasts | KYSE-30 | ND | Differential centrifugation | EX fraction from conditioned medium | Upregulation of 18 miRNA | [129] |
| | Co-culture esophageal carcinoma and fibroblasts | TE1, TE2, TE4, TE6, TE11 | ND | Differential ultracentrifugation | EX fraction from 48-h cell supernatant | miR-1246 | [130] |
| | Esophageal adeno-carcinoma | Het-1A | ND | LSCM, FCM | Het-1A EX fraction | CD95L | [131] |
| Gastric cancer | Gastric cancer | GES-1, MGS-803, SGC-7901 | 24–340 nm | NTA,TEM, IFC | 48 h supernatants fraction | ND | [132] |
| | Gastric carcinoma | SGC-7901 (also co-cultured with HUVEC) | ~100 nm (TEM) | Differential centrifugation; TEM, ELISA, qRT-PCR, functional assays, immunohistochemistry (CD31, anti-VEGF), western blotting (anti-Bim, anti-GAPDH) | Cultural supernatant EX fraction | miR-29a/c | [133] |
| | Gastric cancer | MKN-1,-7,-45,-74 | ND | Differential ultracentrifugation; qRT-PCR, western blotting (tetraspanin-8), RNAi, functional methods | 48 h supernatants MVs fraction | TSPAN8 prognostic marker | [134] |
| | Gastric cancer | SCG-901 | ~50 nm (TEM) | Differential ultracentrifugation and filtration (0.20 mkm); TEM, FCM, functional assays, microRNA microarray, western blotting (CD9, HSP70) | EX fraction from 48 h cultural supernatants | $CD97^+$ | [135] |
| | Gastric cancer | GC415 | Range 60–900 nm (highest conc. 80–120 nm) | Differential ultracentrifugation; FCM, DLS, NTA, AFM, functional assays | Fractions from supernatants (confluent cell cultures) | $CD44^+$, $CD44v6^+$, $CCR6^+$, $HER-2^+$ | [99] |
| | Scirrhous gastric carcinoma cell line and gastric cancer peritoneal lavages | OCUM-2 M, 2 M–D3 | | 0.22 μm filtration and differential ultracentrifugation; miRNA microarray, qRT-PCR | EX fraction from supernatant | miR-320c, miR-1202, miR-1225-5p, miR-1207-5p, miR-7270 signature (validated in peritoneal lavages) | [73] |
| | Duodenal cancer | Primary AZ-521 (HuTu-80), metastatic AZ-P7a | ND | LC-MS/MS, RT-PCR | AZ-521 (HuTu80), AZ-P7a EX fraction | PABP1 | [136] |
| | Gastric cancer | GC-MSC, GCN-MSC, BM-MSC from tissue samples | ND | 0.22 μm filtration and differential ultracentrifugation and ExoQuick™; miRNA microarray analysis, qRT-PCR, functional assays | MSC-derived EX fraction | miR-214, miR-221, miR-222 | [137] |
| | Gastric cancer | SGC7901, HGC27 and gastric epithelial cells | 40–100 nm (TEM) | Differential ultracentrifugation; TEM, western blotting (CD9, CD81, TGFβ, VEGF, N-cadherin, E-cadherin, GAPDH), qRT-PCR, functional assays | EX fraction | ND | [138] |
| | 8 gastric cancer, 5 colon cancer, 9 pancreas cell lines | Most of experiments with gastric cancer AZ-P7a cell line | Defined by IEM | Centrifugation and filtration; immunoelectron microscopy; western blotting (CD29, Alip1/Alix, Tsg101), miRNA profiling and microarray analysis | $CD63^+$ EX fraction after combination of centrifugation and filtration | Let-7 miRNA family | [139] |
| | Gastric cancer | SGC7901, BGC823 | 30–100 nm | EM, western blotting | SGC7901, BGC823 exosomes | Cbl-b, Cbl-c | [140] |
| Pancreatic cancers | Pancreatic stellate cells | Immortalized pancreatic cancer cells | 30–110 nm | Differential ultracentrifugation; TEM, western blotting (CD9, 63, 81, GM130), Real-time PCR, functional assays | $CD9^+$, $CD63^+$, $CD81^+$, $GM130^{-EX}$ fraction from conditioned media | Increased miR451 | [141] |
| | Pancreatic cancer | PC patient-derived cell lines | NTA data | Ultracentrifugation, NTA, western blotting | CD63, TSG101 and ALIX exosomal | ND | [142] |

*(continued on next page)*

**Table 3** *(continued)*

| Cancer type | Cancer subtype | EVs source | EVs size | Detection and purification methods | Subpopulations of EVs | Potential biomarkers | Ref. |
|---|---|---|---|---|---|---|---|
| | | | | | markers confirmed | | |
| | Pancreatic ductal adenocarcinoma (PDAC) | BxPC-3 and HPAF-II | 100 nm | EM, FCM, NTA | PDAC-derived EX fraction | ND | [88] |
| | Pancreatic adenocarcinoma and epithelioid carcinoma | PANC-1, SW1990, BxPC-3 | 30–110 nm | NTA, RT-PCR, FISH | PANC-1, SW1990, BxPC-3 derived EX fraction | RFXAP, miR-212-3p | [143] |
| | PDAC | AsPC-1, BxPC-3, PANC-1 | 50–120 nm | SEM, LSPR-based sensing platform | Cell lines-derived EX fraction | miR-10b | [90] |
| | Pancreatic carcinoma | Panc-1, MIA Paca2, T3M4 | < 100 nm | EM, FCM, UPLC-MS, RT-PCR | Panc-1, MIA Paca2, T3M4 EX fraction | $GPC1^{+}$ | [91] |
| | PDAC | PANC-1 | 40–150 nm | Ultracentrifugation, AcH activity, NTA, DLS | PANC-1-derived EX fraction | ND | [144] |
| | | bxPC-3 SW | | Ultracentrifugation | | miR155 | [145] |
| | Pancreatic adenocarcinoma | INS-1, HPDE PANC-1 | ~100 nm | NTA, EM, CM, SEM | PC-derived EX fraction | CA19-9 and AM | [89] |
| | Pancreatic adenocarcinoma | Paca44, Panc1, BxPc3, MiaPaca2, HPSC and HPDE | < 100 nm | TEM, LC-MS | Paca44, Panc1, BxPc3, MiaPaca2, HPSC and HPDE EX fraction | MET, CIT, MAP4K4, Yes1, DDR1, WEE1, SRC, CDCP1, TCSF | [146] |
| | Pancreatic epithelioid carcinoma | Panc-1 | 30–100 nm | RT-PCR, EM, western blotting | Panc-1-derived EX fraction | miR-203 | [147] |
| | Pancreatic cancer | Panc02 cells, cultivated and injected in mice | 60–100 nm | Magnetic bead-based exosome extraction; AchE-assay, TEM, mRNA detection/measurement | EX fraction purified with magnetic beads coated with biotinylated lectin specific to 2,6-linked sialic acid sandwich | ASPN, Foxp1, Apbb1ip, BC031781, Daf2, Gng2, Incenp | [148] |
| | Pancreatic cancer | AsPC1, BxPC3, Capan1, Capan2, MiaPaca1, Panc1, Pt45P1, 8.18, HD3522, HD3542, HD3577 | | FCM, functional assays | EX fraction, purified by ultracentrifugation | Tspan8, CD44v6, alpha6beta4 | [60] |
| | Pancreatic cancer | BxPC3, MiaPaca2, Panc1 | ND | Ultrafiltration and ultracentrifugation, western blotting, ESI-MS/MS | EX fraction was enriched for EX marker proteins Alix, CD9, Lamp3, syntenin | EGFR | [149] |
| | Rat pancreatic adenocarcinoma | BSp73ASML | ND | Ultracentrifugation, SDS-PAGE, western blotting, MALDI-TOF | EX fraction | CD44v6-dependent exosomes | [55] |
| | Pancreatic cancer | SOJ-6, BxPC-3, MiaPaCa-2, and Panc-1 | 34–45 nm | EM, SDS-PAGE, MALDI-TOF | SOJ-6, BxPC-3, MiaPaCa-2, and Panc-1 nanoparticles | ND | [150] |

understood, however, there are indications that uncoded regions of RNA are involved in this process [65]. Protein and miRNA signatures carried by EVs may represent potential biomarkers as reported by several groups (summarized in Tables 1, 2, 3).

## 3. EV cargo in gastrointestinal cancer

The phenotypic and functional heterogeneity of cancer cells that arises from heritable and stochastic epigenetic and genetic changes increases risk of metastasizing or resistance to drug treatment, and therefore represents one of major challenges in cancer patients treatment [151–152]. Recent research in cancer heterogeneity is adding additional layer of complexity to systemic transfer of extracellular vesicles and their functional content between the cells, which can contribute to tumor progression and influence anti-cancer therapies [153].

### 3.1. EV-associated RNA in gastrointestinal cancer

All major RNA classes are present in EVs [46]. In addition to mRNA and miRNAs [48,154], vRNAs and yRNAs, the degraded products of non-coding and long-coding RNAs have been found in exosomes [155–156].

It has been shown that miRNA dysregulation is involved in the initiation and progression of human cancers although the natural mechanism of this phenomenon is still unclear [157–158]. In normal conditions, these small noncoding, 19 to 22 nucleotide length RNAs participate in regulation of cellular development, differentiation, proliferation, apoptosis, and cancer cell metabolism [158]. Goldie and coauthors [159] demonstrated that exosomes contain a higher proportion of miRNAs compared to other classes of small RNA. It has been shown that exosomes are enriched with miRNA [159], certain types of miRNA are selectively sorted to exosomes and other MPs [160].

However, the details of the mechanism of this selective sorting of miRNAs and miRNA-associated proteins in exosomes and/or MPs are not clear. In mammals, this process includes transcription of miRNA genes into primary miRNAs (pri-miRNAs), and processing by the Drosha complex to produce precursor miRNAs [161]. Pre-miRNA undergoes digestion by the Dicer complex and is sorted to exosomes after becoming mature miRNA via four potential pathways: (1) nSMase2-dependent pathway; (2) miRISC-related pathway that is co-localized with the sites of exosome biogenesis (MTB) and their components (AGO2 protein and miRNA-targeted mRNA); (3) 3′ miRNA sequence dependent pathway, and (4) miRNA motif and sumoylated hnRNPs-dependent pathway (described in detail by [161].

Though uridylation [162] and sumoylation of miRNA [163] are implicated in preferential sorting of miRNA in exosomes, in most cases these targeting sequences are absent in secreted miRNAs [164]. McKenzie and co-authors [165] demonstrated using wild-type and KRAS-mutant colon cancer cells, that KRAS-dependent activation of the MEK-ERK pathway (mitogen-activated protein kinase kinase/extracellular-signal regulated kinase) inhibits sorting of the Argonaute (Ago) 2 dependent miRNA in exosomes. There are early reports that demonstrated the presence of Ago2, a major component of RNA-induced silencing complex (RISC) as well as other proteins responsible for RNA-processing in secreted exosomes [166–167]. Recently, another component of RISC-complex, Y-box protein-1 has been shown to be involved in sorting miRNA to $CD63^+$ exosomes [168]. Surprisingly, the authors did not find evidence of Ago proteins in their isolated exosomal preparations ($CD63^+$ exosomes), which led them to suggest a different route for miRNA egress via exosomes, which they named the chaperone-mediated route as opposed to the Ago2-associated route.

The physiological role of EV miRNA and other cargo has been the subject of discussion from the moment of their discovery in EVs. Though transfer of exosomal miRNA between malignant and non-malignant cells has been proved in numerous studies [48,169–170] (Fig. 2), the functional role of EV-associated miRNAs remains elusive.

Most of the GI cancer-related miRNA studies are based on analysis of tissue and stool samples [171–173] and recently on plasma-derived miRNAs [174–177].

Significant upregulation of miRNA92a, miRNA221, miRNA29a and miRNA17-3p compared to healthy control groups was revealed. Furthermore, Huang and colleagues [174] showed miR-17-3p and miR-92a to be downregulated, giving a potential biomarker for colorectal cancer (CRC) evaluation. Huang and Yu's recent work [78] elucidated the possible mechanism of circulating miRNAs and long non-coding RNAs (lncRNAs) secretion for gastric cancer diagnosis and demonstrated that circulating miRNAs and lncRNAs exhibit higher diagnostic values relative to conventional tumor markers such as CA199, CA125, CA724, CA242, CA50, CEA, and pepsinogen. Another group [177] studied circulating miRNA92a carried by EVs from CRC patient's

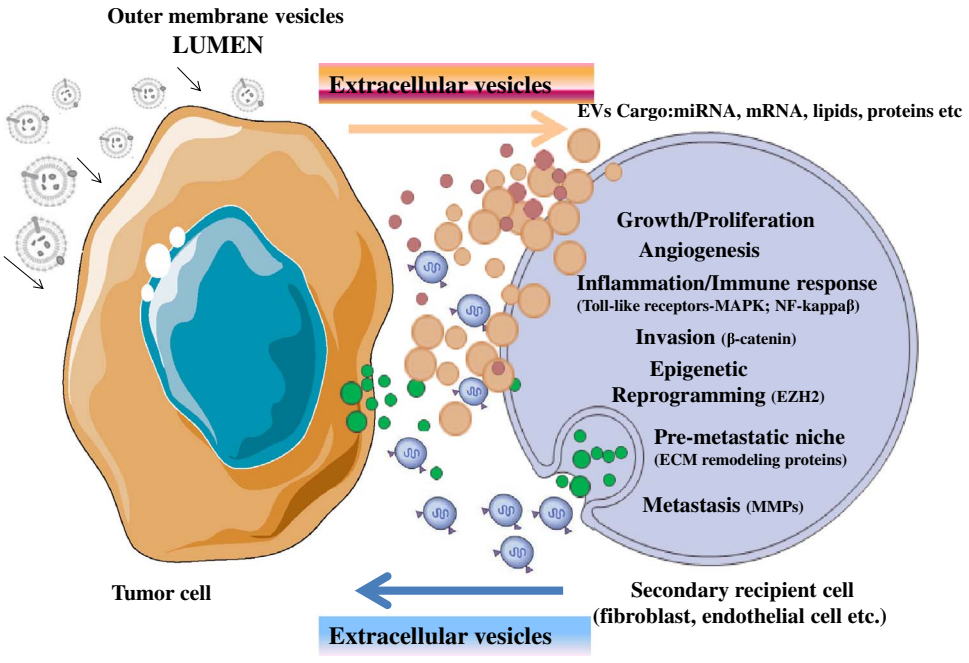


**Fig. 2.** Communication by EVs between lumen, tumor cells and secondary recipient cells (fibroblast, endothelial cells etc.).

plasma and demonstrated high expression levels of miR-92a leading to down regulation of the anti-oncogene Dkk-3. Moreover, secretion of miR-92a–containing EVs by cancer cells into their surrounding environment facilitated angiogenesis.

It has recently been established that gut microbiota has a significant influence on the expression pattern of miRNA of intestine cells including miRNA packed in EVs. In the Dalmasso and co-authors study [178], germ-free mice, lacking natural microbiota, were reconstituted with microbiota from normal special pathogen-free mice leading to the changes in the miRNA expression profile. It was found that after reconstitution nine miRNAs were differentially expressed with four being upregulated: miR-298, miR-200c, miR-342-p. Among these, miR-200c was known to be associated with EVs of highly invasive cancer cells [179–180]. Thus, expression profiling of miRNA in EVs may indicate changes in gut microbiota and a possible predisposition to cancer.

The most remarkable feature of miRNA is its stability in human biofluids, which allows miRNA molecules to operate outside cell borders within hostile environments such as intestinal lumen.

### 3.2. Proteomic studies on gastrointestinal cancer-derived EVs

The underlying mechanisms of protein interactions in EVs and interrelationships between vesicular transport proteins remain to be studied in GI cancers. To date, some authors have evaluated the role and function of the proteomic composition of EVs derived from primary human gastrointestinal cancers and EVs produced by cell lines of gastrointestinal origin [139]. Choi and co-authors [181] described how EVs derived from the HT29 cell line are organized by protein-protein interactions (PPIs) showing that vesicular transport proteins are interconnected via physical interactions and assembled into functional modules. As a result of the study, 957 of the 1261 vesicular proteins were mapped onto the extracellular vesicle PPI network, where 304 of them do not have any PPI in the Human Proteome Reference Database. Also, it has been shown that actin and actin-binding protein ADP ribosylation factors (ARF), such as ARF1and SRC kinases have the essential signaling role in EV biogenesis [181]. Later studies confirmed the role of ARFs, such as ARF6 in exosomal biogenesis [182]. Another study [183] researching serum biomarkers for colorectal cancer metastasis using the secretome approach identified a total of 910 proteins from the conditioned media and 145 differential proteins in SW480 and SW620 cell lines. The differential expression pattern of 6 candidate proteins was validated by Western blot analysis and receiver operating characteristic curve analysis confirmed that serum trefoil factor 3 and growth/differentiation factor 15 could provide a discriminatory diagnostic test for predicting colorectal cancer metastasis. Ji and coauthors [184] found selective enrichment of metastatic factors (MET, S100A8, S100A9, TNC), lipid raft and lipid-raft associated membrane proteins (CAV1, FLOT1, FLOT2, PROM1) and some signal transduction molecules (JASG1, SRC, TNIK) in exosomes derived from metastatic SW60 cells. Enrichment of components of Src-signaling pathway in cancer cell line exosomes identified by proteomic research [181,183] has recently been shown by DeRita and colleagues [185].

### 3.3. Lipids in gastrointestinal cancer-derived EVs

There are a few publications describing a lipid composition of EVs, perhaps because a methodological approach for studying EV lipids is complicated and more difficult than this for EV proteomic analysis. EV membrane lipids have been shown to play an important role in the modulation of a functional response of target cells, formation, secretion and internalization of EVs [35,186–189]. Standard approaches include labeling exosomal and/or other EV subpopulations with fluorescent lipids [34,190], mass-spectrometry based lipidomics [189,191], and recently developed lipid-targeting fluorescent peptide probes [192].

Formation of EVs involves enrichment in certain classes of lipids. Several authors described the enrichment of exosomal fractions with multiple lipid classes including cholesterol (Ch), sphingolipids, phosphatidylserine (PS), gangliosides, free fatty acids [35,188,191,193–194], whereas MPs are enriched in ceramides and sphingomyelins [191]. Lipid changes happen during different steps of EV biogenesis and affect sorting of EV cargo. For example, some proteins are sorted by lipid affinity, such as clustering tetraspanins in tetraspanin-enriched membrane domains (TEMs) associated with gangliosides and cholesterol. TEMs act as sorting machinery for loading growth factors and MHC II molecules into exosomes [195] and cholesterol and ceramides increase exosome release and affect their cargo [186,196].

Exosomes have been shown to contain functional enzymes and important components of lipid metabolism such as phospholipases, hydroxycholesterols, prostaglandins and leukotrienes [34,rev. 35,188,197–198]. Bioactive lipids contained by EVs as a part of their cargo are able to affect the metabolism of target cells (rev. [35]).

To date, the lipidomic characterization of EVs originating from GI tract cancer is limited to only a few publications describing lipid content and/or functional activities involving lipid-mediated pathways induced by exosomes originated from cell lines [150,190,199], while detailed exploration of their functional and causative role remains poorly understood. In one of the ground-breaking publications, Ristorcelli and co-authors [150] reported that the human pancreatic cell line SOJ-6 produced lipid raft-enriched exosomes capable of activating the Notch-1 survival pathway in the cells. To demonstrate the role of exosomal lipids in promoting cellular death, Beloribi and colleagues [190] used SOJ-6 cells and exosome-like synthesized nanoparticles (SELN) composed of lipids typical for lipid rafts. SELN were co-localized with a marker for lipid rafts ganglioside GM1 and Notch-1 on plasma membrane or Notch-1 and Rab5a on early endosomes. Furthermore, Beloribi and co-authors [190] demonstrated a fusion and exchange of SELN lipids with lipid rafts of plasma membrane and endosomal membrane and showed that SELN mimicked detrimental effects of exosomes on the survival of SOJ-6 cells, but not on exosome-insensitive pancreatic MiaPaCa-2 cells. Exploration of lipids as potential cancer biomarkers has only recently begun and depends on technological progress in lipidomic mass-spectrometry. One notable example is the work of Lydic and co-authors [189], who performed a comprehensive, in-depth characterization of a “shotgun” lipidomic profiling of exosomes secreted by LIM 1215 colorectal cell line in comparison with parental. Using novel sample derivatization techniques, coupled with high-resolution “shotgun” mass-spectrometry and targeted mass-spectrometry, they demonstrated that secreted exosome glycerophospholipid compositions are clearly distinct from parental cells indicating that exosome formation/secretion requires unique partitioning of particular lipid classes and subclasses. Comparison of exosome versus cellular lipid profiles reveal > 520 individual lipids in 36 lipid classes and subclasses, as well as substantial lipid remodeling including an increase of sphingolipids, plasmalogen- and alkyl ether-containing glycerophospholipids in exosomes. Importantly, obtained lipidomic results are in broad agreement with publications on exosome content from other cancer types. A deeper analysis may provide insights on lipid exosome role in cancer progression. The potential implication of these studies for diagnostics is yet to be revealed.

## 4. Circulating EVs in gastrointestinal inflammatory disorders and cancer-derived EVs

A number of studies demonstrated that certain circulating EV fractions are increased in patients with GI cancers, compared to patients with inflammatory gastrointestinal diseases such as Crohn's disease (CrD) and inflammatory bowel disease (IBD) [12,13,104,200–204]. However, the amount of circulating microparticles may be also elevated in the active phase of gastrointestinal inflammatory diseases in comparison with healthy controls or cancer patients in remission. Thus, conducted studies on CrD revealed that the total number of circulating MPs was significantly elevated in CrD patients with active processes

compared to healthy controls and CrD patients in remission [201,205]. CrD patients in active phases of disease demonstrated an increased total amount of circulating MPs, particularly pro-coagulant MPs, and MP fractions originating from platelets, erythrocytes, leukocytes and endothelial cells compared to the patients in remission [201].

These elevated populations of MPs may cooperate in the inflammatory process as they induce neutrophil and endothelial activation, monocyte adhesion and recruitment of various inflammatory cells [201,206–207]. Another study in IBD patients found their levels of circulating $TF^+$ MP to be significantly higher than in healthy donors [202]. Interestingly, higher numbers of circulating total, platelet- and endothelial-derived MPs were also observed in patients with IBD in remission as compared to healthy donors supporting the hypothesis of inflammatory cell recruitment [201]. Similar high levels of platelet-derived MPs were demonstrated in earlier studies on IBD patients with increased thromboembolism risk [208–210]. Moreover, recently, Mitsuhashi and co-authors [203] found that intestinal luminal fluid is also a rich source of proinflammatory EVs carrying IL8, IL6, IL10 and TNF markers. The luminal liquid contains high quantities of EVs of different origin (EVs produced by cells structuring intestinal wall, bacterial outer membrane vesicles, EVs coming from parasites and food) (Fig. 3).

EV fraction (< 500 nm size) from colonic luminal fluid aspirates from patients with IBD contained $CD63^+/CD66b^+$ (originated from neutrophils) and $CD63^+/MUC\text{-}1^+$ (originated from epithelium) subpopulations, though no statistical significance with healthy controls was found (8.5% vs 10.8% and 39.5% vs 47.4%). EVs of different origin are partly internalized by cell components of intestinal wall, including cancer cells (illustrated by Fig. 4).

Excess of EVs is released in the circulation. Overall, the increase in the amount of circulating EVs or circulating annexin $V^+$-EVs [96,211] has been observed in many cancer types and in inflammatory diseases (rev. [13]), and may not be considered as a specific diagnostic marker without additional immunophenotyping, discovering and validating disease-specific markers.

## 5. Analysis of potential biomarkers of gastrointestinal cancers in EVs

For the last decade EV components have been considered and intensively investigated as potential cancer biomarkers [66,68,75,80,91,95,169,212]. However, the majority of published studies report that the expression of biomarkers (or combinations of biomarkers) is detected only at the advanced metastatic tumor stage, i.e. when tumors are already detectable by other methods.

Recently, Melo and coauthors [91] described the first diagnostic test based on detecting of a membrane-anchored proteoglycan molecule glypican-1 (GPC1) on EVs. The authors were able to differentiate exosomes isolated from the blood of patients with benign pancreatic disease (BPD), from exosomes isolated from the patients with intraductal papillary mucinous neoplasm (pre-neoplastic lesion) based on the levels of exosomal GPC1. This non-invasive test, as authors claimed, allowed them to distinguish patients with pre-cancerous pancreatic lesions, and to identify patients with late pancreatic cancer (pancreatic ductal adenocarcinoma, PDAC) with 100% certainty. A comparison of the $GPC1^+$ exosome-based test with a standard biomarker for pancreatic cancer (CA-19–9) demonstrated the advantage of using the $GPC1^+$ exosome detection method. The authors analyzed blood from 190 patients with PDAC and 100 healthy controls, however, only a few patients with early pre-neoplastic disease. However, there are already new published results from other group [86] that contradict these findings.

EVs of different origins vary in their stability, but reported serum EVs and EVs associated with DNA may remain stable between 4 °C and room temperature for > 24 h [47], which is important for cancer diagnostics. Significant progress and research interest in circulating miRNAs and other non-coding RNAs could establish them robust diagnostic biomarkers of GI cancers if the feasibility is validated. In the context of cancer biology, miRNA-based signatures of circulated EVs could serve as potential biomarkers for clinical use due to their comparatively long half-life, high sensitivity and specificity, relatively easy accessibility and ability for early cancer detection [213]. A major advantage of miRNA compared to other RNA types, is in its high stability compared to longer mRNA transcripts in most biological samples including fixed and paraffin embedded (FFPE) samples [214–215]. However, during long-term storage their stability may gradually and differentially decrease depending on FFPE tissue block age [216]. miRNA stability in circulation is explained by binding to protein and lipid EV content [217–219].

A number of publications describe microRNA profiling in plasma, serum, and culture supernatants of epithelial cell lines of GI cancers [82,93,94,128,220]. Thus, four important miRNAs, namely, miR-1246,

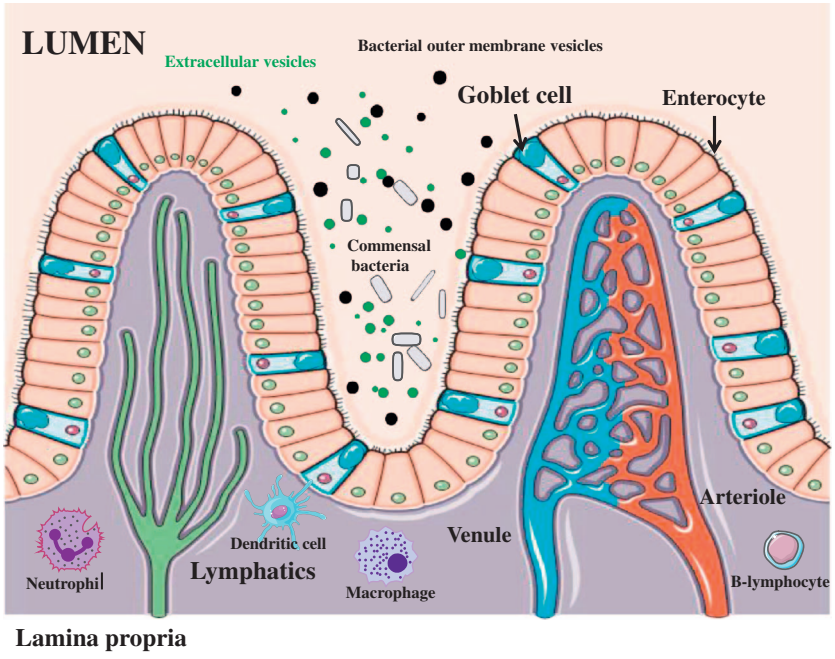


**Fig. 3.** Production of EVs in intestinal lumina (intraluminal EVs; tumor cell-derived-EVs; parasite-derived EVs; bacterial OMVs).

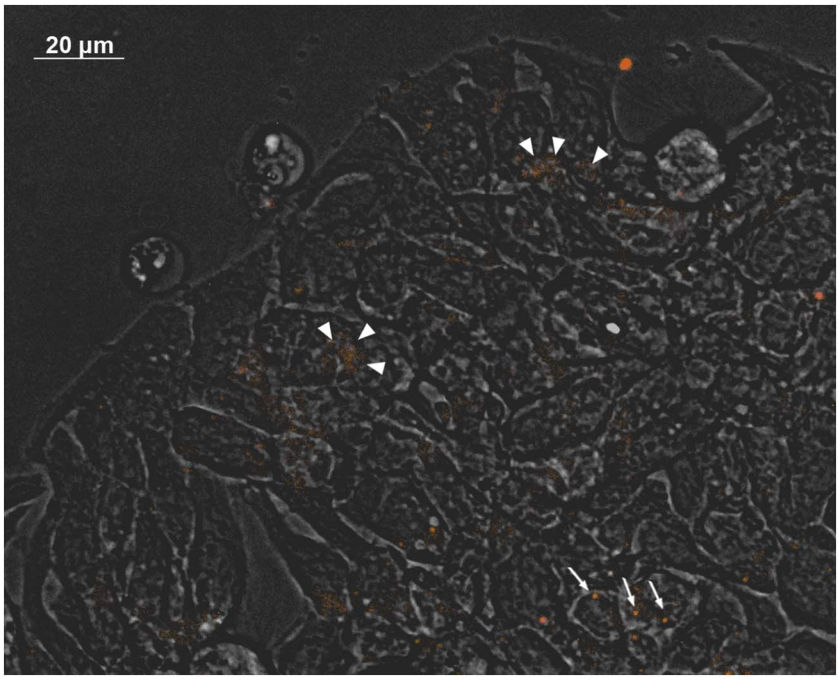


**Fig. 4.** Internalization of cancer-derived EVs by colorectal cancer cell line HT29. EVs from plasma of patients with colorectal cancer were purified, quantitated, labeled by PKH26 and incubated with colorectal cancer cell line HT29 for 18 h at 37 °C. Further cells were imagined with internalized individual EVs (Arrow) and cluster EVs (arrow head) using confocal laser scanning microscope ZEISS LSM780 (objective × 63, 540 to 552 nm band pass excitation filters and 575 to 640 nm band pass emission filters).

miR-4644, miR-3976 and miR-4306, were upregulated in 83% of patients with pancreatic cancer and are considered by the authors [93] to be promising candidates for screening of pancreatic cancer in larger patient cohorts. It is important to note that the profile of miRNAs detected in the EVs is significantly different from that obtained from plasma samples as was demonstrated with miRNA 20a, miRNA 21, miRNA 92a, miRNA 106 and others [221]. Despite the existing optimism that miRNA/miRNA signatures may be considered as perspective biomarkers for colorectal cancer [173,222–224], and other GI tract cancers and gastrointestinal tract inflammatory diseases [225–226], other reports demonstrated that the majority of detected miRNAs are not disease specific [227–228]. Though publications suggesting EV microRNA signatures for cancer grow exponentially throughout the last decade, the suitability for clinical applications is still limited due to absence of endogenous controls to normalize circulating EVs microRNA levels, and conflicting results across the studies.

## 6. EVs and cancer co-evolution

It is now accepted that solid tumors are likely to derive from the co-evolution of neoplastic cells, stromal components, vasculature, and immune cells [229]. As tumors grow in size, an array of molecular modifications aggregates, giving rise to multiple cell subpopulations, each with the ability to proliferate and mutate further. Moreover, neoplastic cell populations are able to regulate the nature of other types of cells in their microenvironment, converting their intrinsic anti-tumoral response into pro-tumoral activity [230–231]. Therefore, a malignant tumor is composed not only of neoplastic cells, which are heterogeneous in terms of genetic and phenotypic features, but also of heterogeneous healthy cell populations participating in anti-tumor immune response and communicating with cells, forming a particular extracellular matrix that supports cancer evolution and progression [151]. EVs are critical mediators of intercellular communication and can orchestrate stroma, tumor microenvironments and tumor heterogeneity influencing the dynamics of disease progression and metastasis outgrowth [232–234], promoting angiogenesis via Egr-1 activation [235], down-regulation of VEGF expression [20], microRNA production [236], and implicating other mechanisms, such as epigenetic regulation of cancer progression.

Longitudinal studies of cancer patients may reveal possible mechanisms of tumor heterogeneity/evolution [237]. Thus, Mege and co-authors [96], compared MPs from patients with CRC, pancreatic cancer, chronic pancreatitis and IBD, and characterized the distribution of different MP cellular origins (platelet-derived MPs (PMPs), endothelial MPs (EMPs), erythrocyte-derived MPs (EryMPs), leukocyte-derived MPs (LeuMPs) and MPs expressing of AnV and different MP antigens (tissue factor (TF), mucin1 (MUC1), podoplanin (PODO), etc.). Besides the differences between patient groups, authors also found microparticle signature changes before and after the “composite” complete remission (CRC), indicating that quantitative and qualitative changes happen in MP signature during cancer evolution.

Another important aspect of cancer evolution is the epigenetic regulation of gene transcription that mediates cancer cell fate, proliferation and differentiation. The initial strategy using aberrant methylation for diagnostics of tumor-specific hypermethylated genes was challenging because chronic inflammation also induced changes in methylation levels [238,239]. The search for early markers of pancreatic cancer also includes epigenetic markers [240]. Thus, DNA methylation of ADAM metalloproteinase and basonuclin in serum appears to have prognostic value for pancreatic cancer [241].

However, tumor evolution and EVs modulating effects on GI cancers are always considered from the point of EVs originating only from eukaryotic cells, and therefore completely neglects EVs originating from human microbiota.

## 7. EVs originated from gastrointestinal cancer and human microbiota

Gut microbiota play a crucial role in the pathogenesis of mucosal inflammation in GI tract disorders. Bacterial, mycobacterial and viral infections are found to be important in IBD pathogenesis [242]. Practically all rodent models of IBD can be mitigated by treatment with antibiotics or by transferring mice into germ-free conditions [243–244]. In mouse models, colitis-associated cancer did not develop in animals treated with antibiotics or germ-free environments [245–246].

The microorganisms that are most frequently associated with cancer development include *Mycobacterium avium* subspecies *paratuberculosis*, adherent enteroinvasive *Escherichia coli*, *Chlamydia pneumoniae* and yeasts, such as *Candida albicans* and *Saccharomyces cerevisiae* [247]. The intestine is normally colonized by approximately 100 trillion

microorganisms [248] comprising 500–1500 different species. Two major commensal groups in the mammalian intestine include *Firmicutes* (gram-positive bacteria) and *Bacteroidetes* (gram-negative bacteria) phyla [249–250] forming approximately 90% of the total microbiome. *Bacteroides* spp. dominates GI microbiota, representing approximately 30% of all bacteria in GI tract, and is most resistant to antibiotics among anaerobes [251]. Patients with GI tract cancers have specific enteric patterns of microbiome associated with an increase of certain bacterial species. Thus, stools derived from CRC patients had increased levels of *Enterococcus*, *Escherichia*, *Shigella*, *Klebsiella*, *Streptococcus*, *Peptostreptococcus*, *Firmicutes*, *Fusobacterium* and *Bacteroidetes* [245,252]. Viljoen and co-authors [253]evaluated important clinico-pathological features in *Fusobacterium* spp. and enterotoxigenic *Bacteroides fragilis* (ETBF), concluding that these bacteria were present at significantly higher levels in late-stage CRC patients in comparison with healthy individuals. *Fusobacterium* spp. is not carcinogenic, but may lead to tumorigenesis indirectly by enhancing inflammation and stimulating cancer cell proliferation [254]. *Fusobacterium* spp. acts by activating FadA adhesion, which triggers colonic epithelial cells (CEC) Wnt signaling and enhances epithelial cell proliferation. The other important bacterial species, ETBF, produces the *B. fragilis* toxin (bft), which is associated with colorectal cancer, diarrheal disease, etc. [255]. It is important, however, to emphasize that α- and ε- clades of *Proteobacteria* including *H.pylori* and another pathogen of digestive tract, *Campylobacter jejuni*, produce altered form of flagellin molecule that is not recognized by Toll-like receptor TLR5, but preserve bacterial motility [256].

As one of the major mechanisms, bacteria representing gut microbiota, release vesicles named outer membrane vesicles (OMVs) for Gram-negative vesicles, or blebs, for Gram-positive bacteria [257]. For simplicity we will use the term bacterial MVs or bMVs to refer to both types of these vesicles. bMVs are 40–300 nm in size, serve as part of the bacterial secretion and transport system and can deliver their cargo (DNA and RNA, protein, lipids and other biologically active molecules) [24,257] to bacterial and/or eukaryotic cells. bMVs are also a rich source of immunomodulating lipopolysaccharides, lipoproteins, peptidoglycans and other bioactive components which are described for many species of Gram-negative bacteria residing in human gastrointestinal tract including *Escherichia coli*, *Helicobacter pylori*, *Fusobacterium nucleatum*, *B. fragilis* and others [253,258–265]. Recently, Chu and co-authors [264], demonstrated that bMVs shed by *B. fragilis* deliver immunomodulatory molecules such as a capsular polysaccharide A (PSA) to intestinal dendritic cells (DC) of mice and activate a noncanonical autophagy pathway requiring IBD-associated genes, *NOD2* and *ATG16L1* for protection from colitis. Consistent with mouse research data, cells from Crohn's disease patients and healthy controls respond to purified PSA and required functional ATG16L1 to induce $CD4^+$ $Foxp3^+$ $IL\text{-}10^+$ T-regulatory cells in response to OMVs from *B. fragilis*. These bMVs require human ortholog IBD associated genes ATG16L1 and NOD2 (the later encodes an intracellular sensor of bacterial peptidoglycan) in order to activate non-canonical autophagy protection pathway against colitis. Moreover, β-lactamases associated with bMVs that produced by *Bacteroides* sp. confer antibiotic resistance not only to the producing organism but also to other commensal bacteria and enteric pathogens (such as *Salmonella typhimurium*) against β-lactam antibiotics [266]. The bMVs have also been implicated in the pathogenesis of gastrointestinal chronic inflammatory diseases including Crohn's disease [263] and *Helicobacter pylori* associated inflammation [261].

## 8. Extracellular vesicles, interspecies communication and molecular mimicry

Gastrointestinal tract is a place where inter-species and even inter-kingdom communication constantly occurs. EVs derived from host eukaryotic cells and from prokaryotic symbiotic and/or pathogenic cells, edible plants [267], fungi and parasitic EVs produced by helminths [268] meet in intraluminal space and interact with intestinal cells. Recently, interspecies communication between nematodes (helminths) and host intestinal cells was reported by Buck and co-authors [269]. Remarkably, *Heligmosomoides polygyrus* secrete miRNA-loaded EVs that are internalized by host mice cells and suppress host immune response. EV secretion was shown for many human parasites, such as trematodes (*Schistosoma mansoni* and *Schistosoma japonicum*) [270,271] and nematodes (*Fasciola hepatica*, *Teladorsagia circumcincta*), and plays an important role in establishing and maintaining parasitic infection [272]. Circulating exosomal miRNAs after internalization by target cells can also act as ligands of Toll-like Receptors (TLRs) [273]. Interestingly, mice TLRs (TLR13) were reported to recognize conserved nucleic acids such as 23S ribosomal RNA molecule of bacterial pathogen *Staphylococcus aureus* [274]. Recent progress has led to identification of a large variety of cytosolic nucleic acid sensors [275]. Further research in this area offers new scenarios to understand complexity that exists in the interaction between organisms on inter-species and inter-kingdom levels.

The intestinal EVs represent a huge reservoir of microbial and parasitic antigens. New evidence demonstrates that bacterial DNA integration and related mutagenesis through lateral gene transfer may occur in cancer cells [276,277]. EVs can serve as a hypothetic vehicle for this transfer, since they carry different types of DNA and RNA. Moreover, protein epitopes can be shared between microbial molecules and self-antigens, and molecular mimicry can lead to the formation of cross-reactive antigens and/or activation T-lymphocytes. Target epitopes can have only distant homologies with initial antigenic triggers [278] and epitope spreading can lead to tissue destruction, apoptosis and concomitant presentation of self- and microbial antigens [279].

**Table 4**
Proteomic analysis of colorectal cancer-derived EVs [123,281–282] and matching protein sequences in commensal and pathogenic microorganisms of GI tract.

| Protein family | Protein found in human colorectal EVs proteome | Microorganism containing matched protein sequence |
|---|---|---|
| Integrins | Integrin-α2 precursor;<br>Integrin-α5-precursor;<br>Integrins α1, 2, 3, 6; β1, 4 | *Helicobacter pylori* (CagL) interacts with α5β3 and α5β1 integrins [283–284];<br>*Candida albicans* (SAP4, SAP5) [285] |
| Regulatory proteins (regulation of actin skeleton) | Tuba-protein | *Listeria monocytogenes* (InIC binds with high affinity to SH3 Tuba domain) [286]) |
| Keratins | 2a, 10, 13, 19, 24, type I, cytoskeletal 9, 14, 18, 19, 20, type II, cytoskeletal 1, 2, 5, 8 | *Enterobacteriaceae* (serine protease autotransporter pet protein binding to K8) [287];<br>Enteropathogenic *Escherichia coli* (keratin-dependent actin reorganization) [288];<br>*Salmonella enterica* (interaction SspC protein with K8) [289];<br>*Salmonella enterica* (interaction SipC protein with K18 [290];<br>*Shigella flexneri* (IpaC binding to K18) [291]; *Streptococcus agalactiae* (Srr protein binding to K4) [292];<br>*Streptococcus parasanguinis* [293–294] |

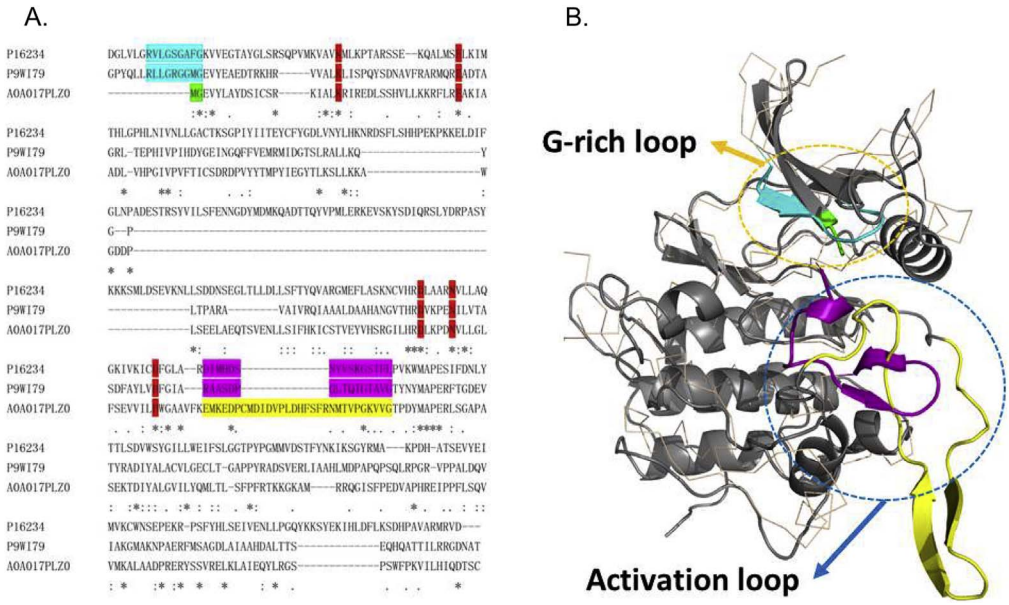


**Fig. 5.** (A) Multiple sequence alignment for three kinase domains from Human, *Mycobacterium tuberculosis*, and *Bacteroides fragilis*, respectively. Left part shows multiple sequence alignment of three kinase domains P16234 (591–983), P9WI79 (13–295), and A0A017PLZ0 (1–295) from Human, *Mycobacterium tuberculosis*, and *Bacteroides fragilis*, respectively. Here P16234 is Platelet-derived growth factor receptor alpha (PDGFR-alpha), whereas P9WI79 and A0A017PLZ0 are Serine/threonine-protein kinase (PknD); (B) Superposition of the kinase domains from Human (*represented in thin ribbon*) and *B. fragilis* (*represented in thick cartoon*). Residues that are critical for kinase activity are shown red in the multiple sequence alignment. For Human and *Mycobacterium tuberculosis*, we show G-rich loop in cyan and activation loop in purple; whereas for *Bacteroides fragilis*, G-rich loop is shown in green and activation loop in yellow (detailed description in Suppl. File 1). From the Fig. 4, the PknD from *B. fragilis* lost the most part of G-rich loop, as well as sequences differ largely at activation loop. Since G-rich loop is critical for ATP-binding, PknD from *B. fragilis* could lose the kinase activity and become a pseudo-kinase. (Methods are provided in Suppl. File 1).

Microbial commensals can induce cross-reactive lymphocytes (rev. [279]) and antibody responses in the gut can be directed against commensal and self-antigens cases being polyreactive in up to 25% [280].

We hypothesized that colorectal cancer proteomes derived from EVs released by GI tract cancer cells may have protein sequences matching gut microbiome organisms. To investigate this, we re-analyzed the colorectal EV proteome studied by the Choi group [123,281,282] and compared it with protein sequences from different commensal bacteria and viruses. As a result, a number of matching microbial sequences was identified (please, see Table 4 for detailed analysis results).

Furthermore, as shown in Fig. 5, we identified the pseudokinase domain in the *B. fragilis* genome with sequence matching platelet-derived growth factor receptor alpha (PDGFR-α). Mutations of PDGF/PDGFRs genes, particularly in kinase and juxtamembrane domain [295-297] can lead to an increased PDGFR signaling and is notably associated with the stage, grade and poor outcomes of various cancers including colorectal cancers [298,299]. Interestingly, while orthologs in *Mycobacterium tuberculosis* possess the characteristic catalytic residue (aspartate), this residue and segment of 16 amino acids is absent in *Bacteroides* spp., so this protein is predicted to be catalytically inactive, but functional pseudokinase [300]. The loss of catalytic activity is mirrored by the acquisition of a potential activation loop (Fig. 5). We hypothesize that the presence of this pseudokinase with activation loop

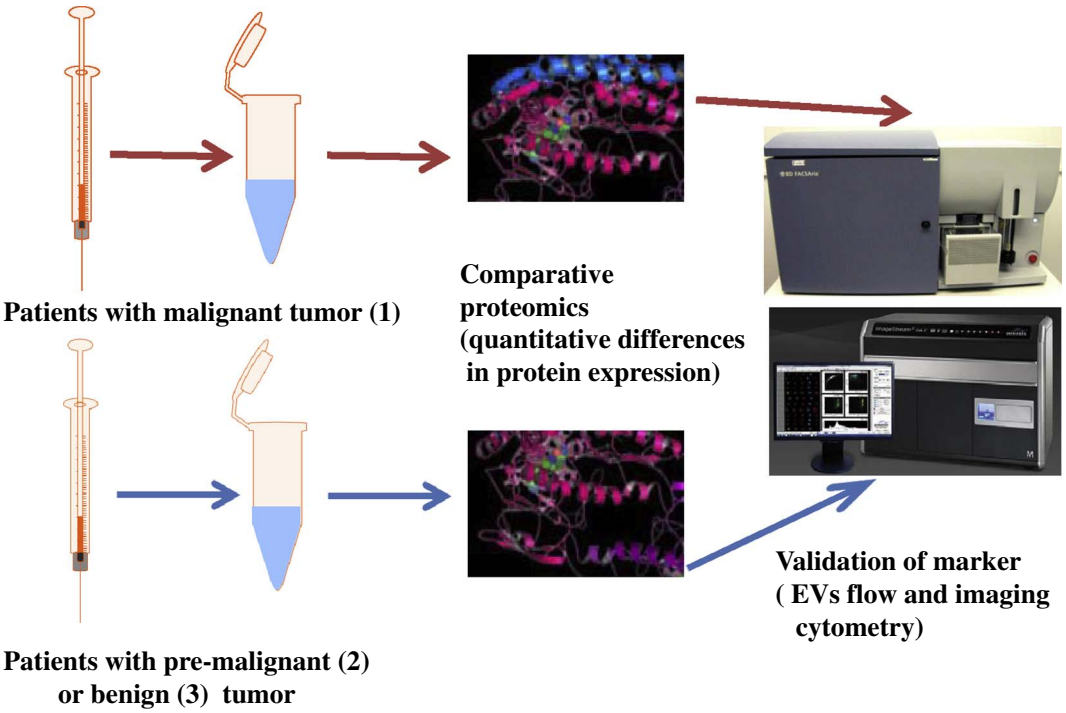


**Fig. 6.** Scheme of identification of new biomarkers highly expressed in EVs. (1) EVs purification; (2) proteomic analysis; (3) identification of hypothetic biomarkers; (4) analysis and validation of hypothetic biomarkers by flow and imaging flow cytometry.

and homology to PDGFR-α in *Bacteroides* spp. may be related to PDGFR-α role in colorectal carcinogenesis.

This novel approach, (i.e. matching protein sequences from eukaryotic EV proteome with the known protein sequences of microbiome gut bacteria) (as well as the opposite – matching bMVs proteome with eukaryotic sequences) helps to identify new similarities between bacteria and eukaryotic proteins and understand their functional role.

## 9. Conclusions and future perspectives

Actively secreted EVs from eukaryotic cells as well as from different microbial, fungi, parasitic species and edible plants in GI tract act as mediators of intracellular and inter-species communication, promote angiogenesis, induce immune suppression, and facilitate tumor cell survival and multi-drug resistance. There is a general consensus that the specific biosignatures of multiple EV biomarkers will be required for diagnostics of GI tract cancers. However, this suggestion recently got challenged when Melo and co-authors developed a simple one-molecule EV-based test for pancreatic cancer [92]. This study was based on the search for proteomic markers highly expressed in EVs originating from patients with advanced tumors and have been identified in early stages of GI tumors. A similar approach can lead to the development of EV-based early diagnosis of different GI cancers, such as CRC (Fig. 6).

The power of EVs is related to their unique protein, RNA, miRNA and DNA profiles, ubiquitous distribution (plasma, saliva, gastric juice, intestinal luminal liquid etc.) and their efficient binding and transfer to target cells [301]. Many efforts have been devoted to EV proteome characterization and protein marker discovery [302,303]. We developed a new approach, consisting of matching protein sequences from eukaryotic EV proteome with known protein sequences to microbiome of gut bacteria as well as matching bMVs proteome with eukaryotic counterparts. We believe that using this strategy, it is possible to identify potential molecular mimicry protein sequences and these findings could be useful for further experimental research of their functional role.

The current challenges in using EV as clinical biomarkers and in researching GI tract cancers include analytical variability of different instruments used for EV detection and variability of sample preparation. The future perspective is the possibility of applying genome editing tools to engineer EVs loaded with “tailored” cargoes. Despite thorough EV research, there is much remaining to be studied to understand the contribution of EVs in GI cancers' pathogenesis and development. Overall, the development of the EV field has the potential for early-stage cancer diagnosis, tracking chemo-resistance and therapeutic efficacy, and tailor-made treatment strategies for GI cancer patients.

## Conflict of interest statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Transparency Document

The http://dx.doi.org10.1016/j.bbcan.2017.06.005 associated with this article can be found, in online version.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbcan.2017.06.005.

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